terminal threonines or serines on proteins may be selectively oxidized to aldehydes.

Small linker molecules may also be used to functionalize proteins and polysaccharides with amino-oxy groups. See, for example, Vilaseca et al., "Protein conjugates of defined structure: synthesis and use of a new carrier molecule," *Bioconj. Chem.* 4:515 (1993); and Jones et al., "Synthesis of LJP 993, a multivalent conjugate of the N-terminal domain of b2GPI and suppression of an anti-b2GPI immune response," *Bioconj. Chem.* 12:1012 (2001).

As is known to those of ordinary skill in the art, amino-oxy, aminooxy, aminoxy, and oxy-amine are all synonymous terms.

F. Indirect Conjugation

As stated above, the conjugation between the first moiety and the second moiety may proceed either indirectly or directly. In certain instances, the process of combining a protein and a polysaccharide may lead to undesirable side effects. In some cases, direct coupling can place the protein and the polysaccharide in very close proximity to one another and encourage the formation of excessive crosslinks between the protein and the polysaccharide. Under the extreme of such conditions, the resultant material can become very thick (e.g., in a gelled state).

Over-crosslinking also can result in decreased immunogenicity of the protein and polysaccharide components. In addition, the crosslinking process can result in the introduction of foreign epitopes into the conjugate or can otherwise be detrimental to production of a useful vaccine. The introduction of excessive crosslinks exacerbates this problem.

Control of crosslinking between the protein and the polysaccharide can be controlled by the number of active groups on each, concentration, pH, buffer

composition, temperature, the use of spacers and/or charge, and other means well-known to those skilled in the art.

For example, a spacer may be provided between the protein and polysaccharide in order to control the degree of crosslinking. The spacer helps maintain physical separation between the protein and polysaccharide molecules, and it can be used to limit the number of crosslinks between the protein and polysaccharide. As an additional advantage, spacers also can be used to control the structure of the resultant conjugate. If a conjugate does not have the correct structure, problems can result that can adversely affect the immunogenicity of the conjugate material. The speed of coupling, either too fast or too slow, also can affect the overall yield, structure, and immunogenicity of the resulting conjugate product. Schneerson et al., *Journal of Experimental Medicine*, 152:361 (1980).

G. Vaccine Compositions

This invention further relates to vaccines and other immunological reagents that can be prepared from the conjugates produced by the method in accordance with the invention. For example, to produce a vaccine or other immunological reagent, the conjugates produced by the method according to the invention may be combined with a pharmaceutically acceptable medium or delivery vehicle by conventional techniques known to those skilled in the art. Such vaccines or immunological reagents will contain an effective therapeutic amount of the conjugate according to the invention, together with a suitable amount of vehicle so as to provide the form for proper administration to the patient. These vaccines may include alum or other adjuvants.

Exemplary pharmaceutically acceptable media or vehicles include, for example, sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil, and the like. Saline is a preferred vehicle when the pharmaceutical

composition is administered intravenously. Aqueous dextrose and glycerol solutions can be employed as liquid vehicles, particularly for injectable solutions. Suitable pharmaceutical vehicles are well known in the art, such as those described in E. W. Martin, Remington's Pharmaceutical Sciences.

The vaccines that may be prepared in accordance with the invention include, but are not limited, to Diphtheria vaccine; Pertussis (subunit) vaccine; Tetanus vaccine; *H. influenzae* type b (polyribose phosphate); *S. pneumoniae*, all serotypes; *E. coli*, endotoxin or J5 antigen (LPS, Lipid A, and Gentabiose); *E. coli*, O polysaccharides (serotype specific); Klebsiella, polysaccharides (serotype specific); *S. aureus*, types 5 and 8 (serotype specific and common protective antigens); *S. epidermidis*, serotype polysaccharide I, II, and III (and common protective antigens); *N. meningitidis*, serotype specific or protein antigens; Polio vaccine; Mumps, measles, rubella vaccine; Respiratory syncytial virus; Rabies; Hepatitis A, B, C, and others; Human immunodeficiency virus I and II (GP120, GP41, GP160, p24, others); Herpes simplex types 1 and 2; CMV (cytomegalovirus); EBV (Epstein-Barr virus); Varicella/Zoster; Malaria; Tuberculosis; *Candida albicans*, other candida; *Pneumocystis carinii*; Mycoplasma; Influenzae viruses A and B; Adenovirus; Group A streptococcus, Group B streptococcus, serotypes, Ia, Ib, II, and III; *Pseudomonas aeroginosa* (serotype specific); Rhinovirus; Parainfluenzae (types 1, 2, and 3); Coronaviruses; Salmonella; Shigella; Rotavirus; Enteroviruses; *Chlamydia trachomatis* and *pneumoniae* (TWAR); and *Cryptococcus neoformans*.

The invention also relates to the treatment of a patient by administering an immunostimulatory amount of the vaccine. The term "patient" refers to any subject for whom the treatment may be beneficial and includes mammals, especially humans, horses, cows, pigs, sheep, deer, dogs, and cats, as well as other animals, such as chickens. An "immunostimulatory amount" refers to that

amount of vaccine that is able to stimulate the immune response of the patient for prevention, amelioration, or treatment of diseases. The vaccines of the invention may be administered by any suitable route, but they preferably are administered by intravenous, intramuscular, intranasal, or subcutaneous injection. For example, carbohydrate-based vaccines can be used in cancer therapy.

In addition, the vaccines and immunological reagents according to the invention can be administered for any suitable purpose, such as for therapeutic, prophylactic, or diagnostic purposes.

The invention also relates to a method of preparing an immunotherapeutic agent against infections caused by bacteria, viruses, parasites, fungi, or chemicals by immunizing a patient with the vaccine described above so that the donor produces antibodies directed against the vaccine. Antibodies may be isolated or B cells may be obtained to later fuse with myeloma cells to make monoclonal antibodies. The making of monoclonal antibodies is generally known in the art (see Kohler et al., *Nature*, 256:495 (1975)). As used herein, "immunotherapeutic agent" refers to a composition of antibodies that are directed against specific immunogens for use in passive treatment of patients. A plasma donor is any subject that is injected with a vaccine for the production of antibodies against the immunogens contained in the vaccine.

EXAMPLES

Example 1: Preparation of an Amino-Oxy Functionalized Protein

The following example illustrates the preparation of an amino-oxy functionalized protein that can be conjugated to a polysaccharide. Bovine serum albumin (BSA) was used as a model protein.

Bis(amino-oxy)tetraethylene glycol was linked to carboxyl groups on bovine serum albumin (BSA) with carbodiimide. Monomer BSA was prepared as described in (Lees et al., *Vaccine* 14:190, 1996). Bis(amino-oxy)tetraethylene

glycol (85 mg) (prepared by Solulink™, MW 361) was made up in 850 µl of 0.5 M HCl. 5 N NaOH was added to adjust to a pH ~4.5. 1 ml of BSA mono (42.2 mg/ml in saline) was added. The reaction was initiated by the addition of 25 µl of freshly prepared EDC (1-(3-dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride, 100 mg/ml in water). After approximately 3 hours, the solution was dialyzed overnight against saline at 4°C. The solution was then made up to 4 ml with saline and concentrated with an Amicon Ultra 4™ centrifugal device (30 kDa cutoff) to ~0.5 ml, and was further desalted on a 1x15 cm G-10 column (Pharmacia) equilibrated with saline. The void volume fraction was then concentrated to ~ 1ml using the Amicon Ultra 4™ device. Using the BCA assay (Pierce Chemical Co), the protein concentration was estimated to be 34 mg/ml BSA. Trinitrobenzene sulfonic acid assay gave an intense red/orange, indicating the presence of amino-oxy group.

Example 2: Preparation of an Amino-Oxy Derivatized Polysaccharide

The following example illustrates the preparation of any amino-oxy functionalized polysaccharide that can be conjugated to a protein, peptide, or hapten.

Pn14 (10 ml at 5 mg/ml in water) was activated by the addition of 40 mg of CDAP (100 mg/ml stock in acetonitrile), followed by triethylamine to raise the pH to 9.4. After approximately 2.5 minutes, 4 ml of 0.5 M hexanediamine (pH 9.4) was added. The reaction was permitted to proceed for about 2 hours. Excess reagent was then removed by dialysis against saline to yield amino-Pn14.

Amino-Pn14 was then reacted with excess NHS bromoacetate at pH 8 and dialyzed against saline in the dark at 4° C. The bromoacetylated Pn14 was concentrated by pressure filtration and then dialyzed against water.

Amino-oxy cysteamine was prepared from bis amino-oxy cystamine by TCEP reduction followed by ion exchange on a Dowex 1X-8 column as follows:

Bis(amino-oxy)cystamine (obtained from Solulink)was made up in 50% NMP/water at 0.1 M. TCEP was made up in water at 0.5 M and 3x molar equivalents of 1 M sodium bicarbonate was added. A 1.5 molar excess of TCEP was combined with Bis(AO)cystamine, and adjusted to pH ~7 with sodium carbonate. After 10 minutes, the mixture was diluted 5-fold into 10 mM bistris at pH 5. The reaction mixture was applied to a 1x3 cm Dowex 1-x8 column that had been washed with 1 M NaCl and equilibrated with 10 mM bistris, pH 5. The reduced amino-oxy cysteamine is found in the flow through of the column.

Amino-oxy cysteamine was added to the bromoacetylated Pn14 and reacted at pH 8 in the dark. The reaction mixture was then concentrated, diafiltered, and then dialyzed against water.

Pn14 concentration was determined to be 9.1 mg/ml by the resorcinol/sulfuric acid method. Using the TNBS assay and amino-oxy acetate as the standard, the amino-oxy concentration was estimated at 0.74 mM, resulting in about 8 amino-oxy groups per 100 kDa of polysaccharide.

Example 3: Preparation of a BSA-Dextran Conjugate

The following example illustrates the preparation of a conjugate vaccine using an amino-oxy functionalized protein and an oxidized polysaccharide. Specifically, the amino-oxy functionalized BSA prepared in Example 2 was linked to oxidized dextran.

Dextran was oxidized using sodium periodate as follows: A 10 mg/ml solution of T2000 dextran (Pharmacia) was made to 10 mM in sodium acetate, pH 5 and then 10 mM sodium periodate (from a 0.5 M stock in water), and incubated at room temperature in the dark. At 1, 5, 10 and 15 min, an aliquot was removed, quenched by the addition of glycerol, and dialyzed against water in the dark. The final concentration of dextran was determined to be about 4.5 mg/ml.

The protein was conjugated to the polysaccharide as follows: 110 µl of each oxidized dextran preparation (1-15 min oxidation) was combined with 15 µl BSA- amino-oxy (0.5 mg each). After an overnight reaction in the dark at room temperature, the samples were analyzed by SDS PAGE (4-12% gradient gel, NuPAGE, Invitrogen). With reference to Figure 1, lanes are conjugates prepared with (A) dex ox 1 min; (B) dex ox 5 min; (C) dex ox 10 min; (D) dex ox 15 min; BSA- amino-oxy only. It is evident that each of the conjugation reactions resulted in high molecular weight material that did not enter the gel. Essentially no unconjugated protein is evident, indicating a high degree of conjugation.

The four conjugates were pooled & fractionated on a S-400HR™ gel filtration column (1x60 cm), equilibrated with saline. The void volume fractions were pooled and assayed for protein and polysaccharide. It was determined that the pool contained 0.21 mg/ml BSA and 0.27 mg/ml dextran. At least 50% of the initial protein and polysaccharide were recovered. Thus, the amino-oxy-protein with oxidized polysaccharide yielded soluble conjugate in excellent yield.

Example 4: Preparation of AO-Functionalized TT.

The following hypothetical example illustrates the preparation of Tetanus toxoid derivatized with amino-oxy groups using a two-step method.

1 ml tetanus toxoid (10 mg/ml) in 2 M NaCl is made to pH 8 by the addition of 50 µl 1 M HEPES, pH 8. The protein is bromoacetylated by the addition of 7 µl of 0.1 M NHS bromoacetate. After a 1 hour incubation, 2 µmoles of aminocysteamine is added. After an overnight reaction, excess reagent is removed by dialysis against 2 M NaCl.

The protein concentration is determined using the BCA assay (Pierce Chemical) and the presence of the amino-oxy group confirmed using TNBS.

Example 5: Preparation of amino-oxy-derivatized BSA using a two-step method

Bromoacetylation of BSA:

4.1 ml of monomeric BSA (48.5 mg/ml) was made to pH 8 by the addition of 400 µl 1 M HEPES, pH 8 and 5.5 ml water. 1 ml of 0.2 M NHS bromoacetate (ProChem) in NMP was slowly added while vortexing. After an overnight reaction at room temperature in the dark, the solution was dialyzed against saline for 2 days, centrifuged and filtered. 10.6 ml of BSA at 15.3 mg/ml was obtained.

Preparation of Amino-oxy cysteamine:

51.5 mg of Bisaminoxocystamine was added to a solution of 56 mg TCEP made up in 1.1 ml 1 M sodium carbonate, 586 µl DMSO, and 586 µl water. After 15 minutes, the TCEP was removed on a 1x5 cm Dowex 1x-8 column, equilibrated with 10 mM Bistris, pH 6. The DTNB positive flow thru was pooled and found to be 22.6 mM thiol.

6 ml was added to the bromoacetylated BSA and the pH adjusted to 8. The reaction was allowed to proceed overnight in the dark, and was then dialyzed for 2 days at 4° C against multiple changes of saline. The amino-oxy BSA was determined to be about 8.6 mg/ml. Reaction of an aliquot with TNBS at pH 8 gave an orangish color, indicating the presence of the amino-oxy group.

Example 6: Use of CDAP to Prepare Amino-Oxy Derivatized Polysaccharide and Amino-oxy Conjugates

This experiment illustrates the use of CDAP to prepare amino-oxy derivatized polysaccharide and amino-oxy conjugates. It illustrates how chemistry other than oxidation can be used to functionalize a polysaccharide with amino-oxy groups.

I. Preparation of an amino-oxy derivatized polysaccharide using CDAP chemistry

A solution of bifunctional amino-oxy reagent was prepared by solubilizing 29 mg of bis-amino-oxy acetate (ethylene diamine) (prepared by Solulink™) in 200 µl 1 M NaAc, pH 5. Dextran was activated using CDAP chemistry as follows. To a solution of 0.5 ml T2000 dextran at 10 mg/ml in water, 25 µl of CDAP (100mg/ml acetonitrile) was added and 30 seconds later the pH was raised by the addition of 25 µl 0.2 M triethylamine (TEA) and three 5 µl of TEA neat.

At 2.5 minutes, the pH was reduced by the addition of 100 µl 1 M NaAc, pH 5. 200 µl of the BisAO solution was then added. After ~30 minutes reaction, the solution was desalted on a 1 x 15 cm P6DG column (BioRad) equilibrated with NaAc buffer (10 mM NaAc, 150 mM NaCl, 5 mM EDTA, pH 5). The desalted polysaccharide was estimated at 1.7 mg/ml dextran, using the resorcinol assay, and about 11 amino-oxy groups/100 kDa dex using a TNBS assay.

II. Preparation of oxidized ovalbumin

To a 0.4 ml solution of ovalbumin (14.4 mg) (OVA), 10 µl of 1 M sodium acetate, pH 5 was added, followed by the addition of 10 µl 0.5 M sodium periodate (in water). After a 15 minutes incubation at room temperature in the dark, the reaction was quenched with the addition of a few drops of 50% glycerol. The reaction mixture was then dialyzed in the dark against NaAc buffer. By adsorption at 280 nm, the concentration of oxidized ovalbumin ("OVA(ox)") was 6.6 mg/ml.

III. Preparation of conjugates and controls

The following solutions were prepared and each was incubated overnight at room temperature in the dark:

A. 500 µl Dex AO (0.85 mg) + 75 µl OVA(ox) + 100 µl 1 M NaAc pH 5.

B. 250 µl Dex AO (0.0.43 mg) + 37.5 µl NaAc buffer + 50 µl 1 M NaAc

C. 250 µl NaAc buffer + 37.5 µl OVA(ox) + 50 µl 1 M NaAc.

Each was then assayed by SDS PAGE and SEC HPLC. Only sample A contained high molecular weight (HMW) material, with ~ 20% of protein conjugated, as estimated by SEC HPLC. Neither B nor C indicated any HMW material by SEC HPLC or SDS PAGE.

Example 7: Use of Cyanogen Bromide to Label Polysaccharide with a Bis-Amino-Oxy Reagent.

This prophetic example demonstrates the derivatization of a polysaccharide with an amino-oxy reagent using cyanogen bromide (CNBr).

Polysaccharide (e.g., Pn-14) is made up at 10 mg/ml in water, and is treated with CNBr at 1 mg per mg of polysaccharide at pH 10.5 for 6 minutes in a pH-stat. The reaction mixture is then reduced to ~pH 7 by the addition of 0.5 M bis-amino-oxy reagent (e.g., bis-AO(EDA). After an overnight reaction, the solution is dialyzed into water and assayed for amino-oxy groups with TNBS, and for carbohydrates with the resorcinol assay. This amino-oxy derivatized polysaccharide is used for conjugation with a carbonyl-containing protein.

According to another embodiment, the CNBr-activated polysaccharide can be reacted with amino-oxy acetate. This will result in a polysaccharide functionalized with carboxyl groups. The carboxyl groups can then be further functionalized and indirectly or directly linked to proteins (with, for example, carbodiimide).

Example 8: Conjugation of Amino-Oxy Derivatized Protein with Oxidized Polysaccharide

This example illustrates the preparation of amino-oxy derivatized protein with the functionalization occuring on the amines. This amino-oxy derivatized protein is then covalently linked to the clinically relevant polysaccharides Neisseria meningididis A and C.

I. Functionalization of protein with amino-oxy groups of a protein on its amines (protein with pendent amino-oxy groups on amines)

Amines on the protein are bromoacetylated and then reacted with a thiol-amino-oxy reagent to produce a protein with pendent amino-oxy groups. Bis(amino-oxy acetate)cystamine 2HCl was prepared by Solulink.™ Monomeric BSA was at 42.2 mg/ml. NHS bromoacetate was obtained from Prochem and made up at 0.1 M in NMP (N-methyl-2-pyrrolidone). The amino-oxy protein was prepared as follows. In each of 2 tubes, a solution of 0.5 ml of BSA (21.1 mg) and 250 µl H_20 + 100 µl 1 M HEPES, pH 8 was prepared. One tube was reacted with a 30 fold molar excess of NHS bromoacetate (93 µl) and the other at a 10 fold molar excess (31 µl).

After about 1 hr, each was made up to 15 ml with sodium acetate buffer (10 mM NaAc, 0.15 M NaCl, 5 mM EDTA, pH 5) and concentrated to about 200 µl using an Amicon Ultra 15™ device (30 kDa cutoff).

Amino-oxy acetate cysteamine was prepared as follows:

To a solution of 9.8 mg of Bis(AOAc)cystamine (prepared by Solulink™) in 114 µl 1 M sodium acetate + 114 µl NMP, 22.8 µl of 0.25 M TCEP in 1 M HEPES, pH 8 was added as a reducing agent. After 1 hour, the partially reduced amino-oxy thiol reagent was added to each of the bromoacetylated BSA preparations, the pH was adjusted to about pH 8 and the reaction allowed to proceed overnight in the dark at 4°C.

Each was desalted using the Amicon Ultra 15™ device by making volume up to 15 ml with NaAc buffer and centrifuging. The desalting process was repeated four times. The final volume was about 200 µl and was then made up to about 1 ml with NaAc buffer. This product was termed BSA-S-AO. By adsorbance at 280 nm, the 30x prep was determined to be 29.8 mg/ml and the 10x prep 24.8 mg/ml.

II. Preparation of oxidized Neisseria meningiditis polysaccharide A and C (Neiss PsA and Neiss PsC)

Neiss PsA and PsC were solubilized overnight at room temperature at 10 mg/ml in water and then stored at 4°C. 50 µl of 1 M sodium acetate, pH 5, was added to 1 ml of each polysaccharide solution, followed by the addition of 25 µl 0.5 M sodium periodate (0.5 M in water). After 10 minutes in the dark at room temperature, each was dialyzed 4 hours against 4 l water. Each was then made up to 4 ml with water and further desalted using an Amicon Ultra 4™ device (30 kDa cutoff). Using the resorcinol assay, the oxidized Neiss PsA was determined to be 12.1 mg/ml and the oxidized Neiss PsC was 17.8 mg/ml.

III. Conjugation of BSA-S-AO with oxidized Neiss PsA and PsC

The following mixtures of BSA-S-AO and oxidized PsA and PsC were prepared.

Conjugate	µl Ps	µl BSA	1 M NaAc pH 5
BSA10x-PsA	175 µl (2.1 mg)	134 µl (4 mg)	25 µl
BSA30x-PsA	175 µl (2.1 mg)	161 µl (4 mg)	25 µl
BSA10x-PsC	175 µl (3.1 mg)	134 µl (4 mg)	25 µl
BSA30x-PsC	175 µl (3.1 mg)	161 µl (4 mg)	25 µl

After an overnight reaction at room temperature in the dark, conjugates were assayed by SDS PAGE using a Phast gel (8-25%)(Pharmacia) under reducing conditions. With reference to Figure 2, from left to right the lanes are BSA30x-PsA, BSA30x-PsC, BSA30x, BSA10x –PsA, BSA10x-PsC, BSA10x. It is seen that there is a significant amount of high molecular weight materials that did not enter the gel, indicating that conjugation of the protein to the polysaccharide occurred.

The PsA conjugates were pooled and fractionated by gel filtration on a S-400HR column (1x60 cm, Pharmacia), equilibrated with saline. Similarly, the PsC conjugates were pooled and fractionated. Approximately 1 ml fractions were collected and assayed for protein (by absorbance) and for carbohydrate using the resorcinol assay. The results are provided in Figure 3.

For the PsC conjugate, tubes 18-22 were pooled and for the PsA conjugate, tubes 19-23 were pooled and examined by SDS PAGE using reducing conditions.

With reference to Figure 4, the BSA-Neiss PsC conjugate is on the left and the PsA conjugate is next to it. On the right is the molecular weight standard. A small amount of free BSA is observed in each, indicating incomplete separation of the conjugated and free protein. Each contains a significant amount of conjugated high molecular weight material that did enter the gel.

Example 9: Preparation of (BSA-Levulate)-Amino-Oxy-Pn14 Conjugate

This example illustrates the reaction of an amino-oxy group with a ketone and shows that this can be used for the formation of conjugates and, more specifically, the preparation of (BSA-Levulate)-Amino-oxy Pn14.

NHS Levulate was obtained from Solulink and made up by solubilizing 5.1 mg in 100 µl NMP. This was slowly added to a vortexed solution of 200 µl BSA at 48.5 mg/ml, 200 µl water, and 100 µl 1 M HEPES, pH 8. After an overnight reaction, the mixture was diafiltered using an Amicon Ultra 15 device, (30 kDa cutoff). The final volume was 0.5 ml. This product is BSA-LEV

100 µl of BSA-LEV was combined with 300 µl of amino-oxy Pn14 (4.5 mg/ml Pn14) and incubated for several days in the dark. The conjugate and the individual components were assayed by SEC HPLC using a Superose 6 column (Pharmacia). The conjugate was then fractionated on an S400HR column. Protein was assayed using the Bradford dye method, and polysaccharide with the